

SMOKERS' MELANOSIS

C. ANDERS HEDIN



Malmö 1986

*Diss. Q41
Lund*

Organization LUND UNIVERSITY Department of Oral Pathology School of Dentistry Carl Gustavs väg 34 S-214 21 Malmö, Sweden	Document name DOCTORAL DISSERTATION	
	Date of issue November 24, 1986	
	CODEN: LUODD(ODOP-1001)/1-62/1986	
Author(s) C. Anders Hedin	Sponsoring organization Faculty of Odontology, University of Lund, Malmö, Sweden	
Title and subtitle Smokers' Melanosis. An epidemiologic, morphologic and experimental study of oral melanin pigmentation caused by tobacco.		
Abstract The prevalence and regional distribution of a clinically visible oral melanin pigmentation was studied. The aim was to investigate an observed correlation with tobacco smoking. The presence of melanin was confirmed by light and electron microscopy. The morphology of melanocytes and keratinocytes, the number of melanin-containing keratinocytes, and the diameter of their melanosome complexes were studied. Melanocyte stimulation by nicotine was investigated by studying the movement of melanosomes in the cytoplasm of amphibian melanocytes by light microscopy and spectrophotometry. In the population, 9.9-14.9 % of the individuals had oral melanin pigmentation. Among non-tobacco users 3.0 % were pigmented and among smokers 21.9-30.9 %. The pigmentation was most common in the mandibular gingiva and buccal mucosa, among female smokers, and among smokers who had been smoking for a long time. Only about 3 % were pigmented among individuals who had stopped smoking. A linear correlation was found between the number of pigmented individuals and the number of cigarettes consumed ($r = 0.98$). The morphology of the studied cells was normal. A larger number of keratinocytes contained melanin in pigmented smokers and non-smokers as compared to non-pigmented non-smokers. Melanosome complexes in pigmented individuals were larger than those earlier reported from human oral mucosa, and could reach 3.2 μm . Amphibian melanocytes were activated by nicotine. The stimulation was non-toxic and was not blocked by propranolol, and is therefore not mediated via β -adrenergic receptors. Tobacco smoking is the main cause for the observed oral melanin pigmentation, and was therefore given the name "smokers' melanosis". It is a non-specific cell reaction caused by components in tobacco. The increased amount of melanin in the oral epithelium of smokers may act as a trap for tobacco related polycyclic compounds and free radicals.		
Key words Beta adrenergic receptors, Caucasoid, chromatophores, connective tissue, dendrites, epithelium, gingiva, isoproterenol, keratinocytes, melanin, melanocytes, melanosomes, nicotine, Ranidae, sex, smoking, snuff, time factor, tobacco.		
Classification system and/or index terms (if any)		
Supplementary bibliographical information		Language English
ISSN and key title		ISBN 91-7900-131-9
Recipient's notes	Number of pages 62	Price
	Security classification	

Distribution by (name and address) C. Anders Hedin, Specialisttandvården, Regional Hospital, S-631 88, Eskilstuna Sweden.

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Anders Hedin

Date October 29, 1986

From the Department of Oral Pathology, School of Dentistry,
University of Lund, Malmö, Sweden.

SMOKERS' MELANOSIS

An epidemiologic, morphologic and experimental study
of oral melanin pigmentation caused by tobacco.

AKADEMISK AVHANDLING

som med vederbörligt tillstånd av Odontologiska Fakulteten vid Lunds
Universitet för avläggande av odontologie doktorsexamen kommer att
försvaras offentligt i aulan, Tandläkarhögskolan, Malmö, fredagen den
19 december 1986 kl 09.15

av

C. Anders Hedin

leg. tandläk.

Malmö 1986

SMOKERS' MELANOSIS

An Epidemiologic, Morphologic, and Experimental
Study of Oral Melanin Pigmentation
Caused by Tobacco.

by

C. ANDERS HEDIN



Malmö 1986

Doctoral thesis from the Department of Oral Pathology, School of Dentistry, University of Lund, S-214 21 Malmö, Sweden.

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Printed in Sweden by AWJ Länstryckeriet AB, Nyköping 1986
ISBN 91-7900-131-9

The future existence of man depends on our ability to distinguish the early signs of biologic alarm, to ask why, to investigate and to prevent damage to the living organism.

TO MY FAMILY

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

- I Hedén CA: Smokers' melanosis. Occurrence and localization in the attached gingiva. *Arch Dermatol* 1977; 113:1533-1538.
- II Axéll T, Hedén CA: Epidemiologic study of excessive oral melanin pigmentation with special reference to the influence of tobacco habits. *Scand J Dent Res* 1982; 90:434-442.
- III Hedén CA: Specimen holder improvements for the study of amphibian skin pigmentation. *J Microscopy* 1983; 132:125-128.
- IV Hedén CA, Larsson Å: The ultrastructure of the gingival epithelium in smokers' melanosis. *J Periodont Res* 1984; 19:177-190.
- V Hedén CA, Larsson Å: In vitro activation of amphibian dermal melanocytes by nicotine. *Scand J Dent Res* 1986; 94:57-65.
- VI Hedén CA, Larsson Å: Large melanosome complexes in the human gingival epithelium. *J Periodont Res* 1986; in press.

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ABBREVIATIONS AND EXPLANATIONS

cAMP	Cyclic adenosine monophosphate.								
Dendrites	Cytoplasmic projections on melanocytes.								
Epithelial melanin unit	A melanocyte in functional contact with epithelial cells.								
EPR	Endoplasmic reticulum.								
Frequency	A statistical expression for the presence of an oral lesion in a population, when the number of individuals having the lesion are divided by the total number of those examined.								
ICD-DA 1978	International classification of diseases; application to dentistry and stomatology.								
Index of determination	A statistical expression for the square sum of the correlation coefficient r .								
Iridophore	An amphibian chromatophore.								
Keratinocyte	The main cell building up keratinizing epithelium in skin and gingiva.								
LMC	A melanosome complex (MC) larger than $1.0\mu\text{m}$. For definition see paper VI.								
Malpighian cell	The main cell of the germinative layer of the oral mucosal epithelium.								
Maturation of melanosome	The melanin deposition on premelanosomes.								
MC	Melanosome complex.								
Melanocyte	Dendritic cell which produces melanin. It has a clear cytoplasm and no desmosomes.								
Prevalence	A statistical expression for the presence of an oral lesion in a population, when due consideration is given to the number of lesions found and the frequency of participation in different demographic groups.								
Significance levels	In the present studies the following levels were used: <table style="margin-left: auto; margin-right: auto;"> <tr> <td>$0.05 < p$</td><td>not significant</td></tr> <tr> <td>$0.01 < p < 0.05$</td><td>*</td></tr> <tr> <td>$0.001 < p < 0.01$</td><td>**</td></tr> <tr> <td>$p < 0.001$</td><td>***</td></tr> </table>	$0.05 < p$	not significant	$0.01 < p < 0.05$	*	$0.001 < p < 0.01$	**	$p < 0.001$	***
$0.05 < p$	not significant								
$0.01 < p < 0.05$	*								
$0.001 < p < 0.01$	**								
$p < 0.001$	***								
Smokers	In these studies only individuals with one tobacco habit were included in the calculations.								
Uvea	The pigmented strata on the inner surface of the iris in the eye.								
Xantophore	An amphibian chromatophore.								

INTRODUCTION

TOBACCO AND ORAL MELANIN PIGMENTATION

During early 20th century a correlation was observed between the use of tobacco and discoloration of the oral mucosa. In tobacco-chewers black or greyish and diffusely demarcated areas were seen within leukoplakias of the buccal mucosa. Outside this area more demarcated blackish-brown to bluish-grey multiple pigmentations, a few to several mm were also found. Tissue from one patient was histologically examined and showed yellow-brown pigment granules in the deepest part of the epithelium, and in the upper part of the connective tissue (Pichler 1916). A patient with a similar clinical pigmentation was studied by Kistiakowsky (1931). In an Italian who was a smoker since 3 years, a blue to black gingiva was noted, which during the following smoke-free 6 months nearly retained its normal pink color. After the patient's taking up smoking again the pigmentation reappeared within 2 months. The discoloration of the gingiva was stated not to consist of melanin, but to be caused by an accumulation of a color-substance in the tissue.

In some of the few early studies on oral melanin pigmentation, it was noted that some pigmented individuals were smokers (Reiche 1924, Stempel & Armuzzi 1924). The pigmentation was supposed to be caused by "Provozierende Momente z.B. Reizungen durch cariöse Zähne, durch Rauchen" or by an inflammation due to the chemical trauma from tobacco (Jadassohn 1926, Monash 1932). In Greenland tobacco-chewers using tobacco-ash, so called "imakut" or "eroq", a greyish-white or bluish-white discoloration was seen in the region where the tobacco-ash was in contact with the buccal mucosa (Pedersen 1938). Melanin was first mentioned in a later epidemiologic study on oral mucosal lesions in Greenland tobacco-chewers (Jacobsen 1968).

The first study including a histologic examination was performed on Bantus (van Wyk 1970). Here it was stated that "neither is it possible to test if the irritation from noxious habits such as smoking and chewing tobacco . . . is the cause (to melanin pigmentation)". In Indian reverse smokers palatal melanin pigmentation seems to be more frequent (Mehta et al. 1977). In Sweden dark localized discolorations did not show any correlation to tobacco habits (Carlson 1971). In reviews published during the last decades, and dealing with the influence of tobacco on oral mucosa, or with the etiology of oral melanin pigmentation a correlation between the use of tobacco and oral melanin pigmentation is not mentioned.

ORAL MELANIN PIGMENTATION IN DIFFERENT ETHNIC GROUPS

The distribution of oral melanin pigmentation has been studied in several ethnic groups without correlating to tobacco habits. In these studies a uniform definition of the pigmentation studied, and a detailed clinical characterization of the lesions is often missing. However, the clinical description mostly makes it clear that oral nevus and malignant melanoma are excluded. Thus, in most studies the described melanin pigmentation will fit with the concept of "excessive oral melanin pigmentation NOS" which includes melanotic macules, 528.96 (ICD-DA 1978). Studies show that oral melanin pigmentations are most common in ethnic groups with heavy skin melanin pigmentation, but also that among these there are individuals without any oral pigmentation (van Wyk 1970).

The pigmented areas in light skinned individuals are described as non-elevated, lightly brown, irregularly demarcated spots of 1-4 mm, most often situated in the central part of the attached gingiva. In dark colored individuals the pigmentation of the oral mucosa is dark brown, brownish black or purplish black. The separate areas confluence to continuous stripes in the central part of the gingiva, but are also seen more or less often in all oral mucosal regions (Monash 1932, Dummett 1945).

In Australian Aborigines, American Negroes and Brazilians with negroid heredity, this pigmentation is observed already in newborn and very young children (Monash 1932, Brown 1964, Eleutério 1969). The number of pigmented individuals is higher among older individuals, and adult black Americans are pigmented in 44-95 %, Australian Aborigines in 90 % and so are nearly all Bantus (Dummett 1945, Brown 1964, van Wyk 1970). Caucasians are pigmented in about 10 % (Axëll 1976). The frequency of pigmented individuals in some ethnic groups is presented in page 10.

THE INFLUENCE OF TOBACCO ON THE ORGANISM

The tobacco smoke consists of a gas- and a particle phase. In the gas phase nitric oxide, carbon monoxide, argon, methane, cyanide, ammonia, acrolein and croton aldehyde are found. In the particle phase, also called the tar, nicotine, benzo(α)pyrene, and a large number of other hydrocarbons are present (Akin et al. 1976). Benzo(α)pyrene is a potent factor in burned tobacco, but nicotine is an active substance in tobacco smoke as well as in cold tobacco.

Nicotine, L-1-methyl-2(3-pyridyl)pyrrolidine, is isolated from the leaves of the tobacco plant *Nicotiana tabacum*. It is one of the most potent poisons, with well known neurogenic effects. Thus, in a low dose nicotine has the same effect on autonomic ganglia as acetylcholine, and shows a cholinomimetic stimulation. In large doses the reverse effect is achieved, i.e. a blockade (Volle & Koelle 1970). In small doses nicotine stimulates the production of catecholamines from the adrenal medulla, free nerve endings, and from chromaffine tissue in different organs (Andersson et al. 1985). This sympathomimetic effect of nicotine, which can be shown by catecholamine blockers, is responsible for the well known contraction in peripheral arterial vessels, with an elevated blood-pressure as a result. Nicotine can pass the blood-brain barrier, which results in stimulation of nicotine-like receptors in the brain (Edvinsson et al. 1977). This finding can explain the stimulatory feelings experienced when using tobacco.

ORAL PIGMENTATION IN SOME ETHNIC GROUPS

Ethnic group	Age	Frequency of pigmented individuals %	Pigmented region	Author
Australian Aborigines	1/2-78	100	Gingiva, lips, cheeks, palate	Brown 1964
American Negroes	0-65	95	Gums, cheeks, hard & soft palate, tongue floor of mouth	Monash 1932
	17-37	72	Gums, buccal mucosa	Dummett 1945
South African Bantus	adults	100	Gingiva, cheeks, hard & soft palate, lips	van Wyk 1970
Brazil negroids	5-14	93	Gingiva, hard palate, buccal mucosa	Eleutério 1969
Londoners colored	adults	38	Buccal mucosa *)	Fry & Almeyda 1968
Greenland Inuits	5-70	98	Gingiva, buccal mucosa	Jakobsen 1968
Israel dark skinned	6-adult	75	Gingiva, buccal mu- cosa, soft palate	Steigmann 1965
Brazil yellow skinned	5-14	70	Gingiva	Eleutério 1969
Japan	1-77	43	Gingiva *)	Ando et al. 1956
Israel light skinned	18-90	25	Gingiva, buccal mu- cosa, lips, floor of mouth, palate, tongue	Gorsky et al. 1984
Londoners Caucasians	adults	5	Buccal mucosa *)	Fry & Almeyda 1968
Brazil white skin	5-14	13	Gingiva	Eleutério 1969
Sweden Caucasians	15- adults	10	Gingiva, buccal mucosa, lips	Axéll 1976

*) The study restricted to this region.

Half of the nicotine in a cigarette will be present in the tobacco smoke. Most of this may be resorbed via the lungs during inhalation, but only one third via the oral mucosa. In a standard cigarette the tobacco smoke gives between 18.4–55.1 ng nicotine per ml blood (Benowitz et al. 1982). The concentration of nicotine in saliva of non-smokers is 36 nM/l, compared to 939–5589 nM/l in smokers (Feyerabend et al. 1982). At an acid pH, the resorption of nicotine through the oral mucosa is low (Kozlowski & Kleiman 1978). This is the situation during cigarette smoking. Smoke from cigars and pipe tobacco is often alkaline, and in these situations the oral nicotine absorption is elevated. This explains why most cigar and pipe smokers achieve a nicotine stimulation without inhalation of the smoke. In the blood as well as in acidic conditions nicotine is metabolized to cotinine, which is present in the urine. This metabolism is most extensive in smokers and is higher in women compared to men (Beckett et al. 1971). The halftime for nicotine is 100–120 min, but during the time of active tobacco consumption the level of nicotine rises in the body, and is still present in biologically active amounts during the whole night (Benowitz et al. 1982).

Tar painting on skin is known to produce skin pigmentation (Lip-schütz 1924). Such painting activates melanocytes and produces pigmented tumors, called melanoma (Pawlowski & Lea 1983). Also benzo(α)pyrene activates melanocytes seen as an enhanced DOPA-activity in mouse skin after 6 days (Iwata et al. 1981). Although benzopyrene probably is the most potent melanocyte stimulator in tobacco, also nicotine is of interest. Smoking of nicotine-containing tobacco stimulates the adenylate cyclase system in the human organism, while smoking of nicotine-poor tobacco does not give this effect (Takahashi et al. 1977). It is known that adenylate cyclase stimulation and cAMP is necessary for activation of melanocytes and melanogenesis and for the transfer of melanin to keratinocytes. These observations indicate that nicotine could perhaps locally influence the melanin-producing system of the oral mucosa.

THE INFLUENCE OF TOBACCO ON THE ORAL MUCOSA

Several oral lesions are associated with the use of tobacco, for instance pipe smoking with nicotine stomatitis, cigarette smoking with hyperkeratosis of the buccal mucosa, and snuff with snuff dippers lesion in the vestibular mucosa. Leukoedema is common in smokers as well as the plaque type of lichen planus. Tobacco induced leukoplakias are at risk to develop into carcinomas (Pindborg et al. 1967, Axëll et al. 1976, van Wyk 1976, Neumann-Jensen et al. 1977, Pindborg et al. 1977). Cigarette tar in combination with high temperature may experimentally give keratotic lesions on rat lip mucosa, but oral cancer is difficult to produce experimentally with tobacco (Bastiaan & Reade 1980).

Benzo(α)pyrene may be metabolized to carcinogens by the enzyme aryl hydrocarbon hydroxylase (AHH). Oral cancer is associated with high AHH levels (Andréasson et al. 1982). Nicotine in combination with nitrite develops into nitroso-nornicotine (NNN) in both cold and burned tobacco, and saliva enhances the toxicity of NNN. NNN is known to promote tumor growth (Hoffmann et al. 1976).

Wound healing in skin and oral mucosa is impaired in smokers, and may partly be due to a local vasoconstriction (Shuler 1968, Mosely et al. 1978, Sweet & Butler 1979, Clarke et al. 1981). A lower resistance against periodontitis is also found among smokers. This is noted clinically by a lower gingival inflammation i.e. less bleeding, less inflammatory exudate from pathologic pockets, but a more extensive alveolar bone loss. This is in agreement with the finding of vasoconstriction in the oral mucosa, disturbed PMN-leukocyte activity, lowered IgG and IgA levels, and the possibility of a generally lower bone quality in smokers (Kraal et al. 1977, Ferson et al. 1979, Hedin et al. 1981, Rungren & Mellström 1984, Preber & Bergström 1985).

MELANOCYTES AND KERATINOCYTES IN THE ORAL MUCOSA

Melanocytes are present in the basal epithelium of skin and oral mucosa in man, other mammals, and also in the skin of amphibians

(Schroeder & Theilade 1966, Zelickson 1967, Taylor & Bagnara 1972). These cells produce cigar-shaped protein structures on which melanin is precipitated, called melanosomes or melanin granules (Schroeder 1969 A). The melanin production starts when the melanocyte is stimulated through a local influence on the skin surface by external factors such as UV-light, but also by endocrine factors such as hormones or by genetic influence. Well known melanocyte stimulators are the hormones MSH and ACTH. Also isoproterenol and other adrenergic agents acting via the specific β -receptor on the cell surface, stimulate the melanocyte (McGuire 1972). This is initiated via the adenylate cyclase system and starts the melanosome production, melanin deposition, elongation of dendrites, movement of melanosomes out into dendrites, and the transfer of melanized melanosomes to the keratinocytes (Wikswa 1973). In the skin melanin serves as a sun protector. Melanosomes are distributed within the keratinocyte cytoplasm according to their size. Large melanosomes are dispersed as single granules, while small, less melanized melanosomes are grouped 3-5 granules together within or without a limiting membrane. Most melanosomes are disintegrated in the suprabasal epithelium by lysosomal enzymes, making melanin ultrastructurally invisible in higher cell layers (Wolff 1973).

Because of an observed clinical connection between tobacco smoking and oral melanin pigmentation, the following epidemiologic, ultrastructural and experimental studies were performed.

AIM OF THE STUDIES

The aim of the present investigations was to study the correlation between tobacco smoking and oral melanin pigmentation through:

- analyzing epidemiologic data
- a morphologic and histochemical indentification of the distribution and amount of melanin i.e. melanosomes
- an experimental documentation of one of the main components of tobacco — nicotine — to be a stimulator of the melanin producing cell, the melanocyte.

MATERIAL AND METHODS

CLINICAL STUDIES (I, II, IV & VI)

Subjects

The initial observation concerning a correlation between oral melanin pigmentation and tobacco smoking was made in the gingiva. Although also other oral mucosal areas were observed to be pigmented, the first study was restricted to the gingiva (I), while a more extensive investigation (II) covered all oral mucosal regions.

The frequency of melanin pigmentation in the attached gingiva was studied in 121 randomly selected patients (53 men and 68 women) between the age 21–75 years, from the patients referred to the Dental School, University of Umeå, in the north of Sweden. The periodontal status of these patients varied between gingivitis and advanced periodontitis. In order to study the frequency of melanin pigmentation in the gingiva of individuals with good gingival health, 93 female dental nurse students were examined. They comprised all the students of three subsequent courses of dental nurse education. Their average age was 21.1 years, and their gingival status was excellent. Minor areas of edema or erythema, corresponding to gingivitis, were however observed. To study the localization of gingival pigmentation in a large number of pigmented patients, and a possible connection with tobacco, 71 individuals (23 men and 48 women) between 15–70 years of age with clinically visible gingival melanin pigmentation, were selected from the referred patients during a one year period.

To investigate the correlation between excessive oral melanin pigmentation in all oral mucosal regions, and different tobacco habits, a study was performed on a general population (II). It consisted of 20 333 individuals, 10 036 men and 10 297 women, aged 15 years and older and living in central Sweden. The data from this population was collected by Axéll (1976).

To study the ultrastructure of melanocytes and keratinocytes, to correlate the histologically demonstrable distribution of melanin in keratinocytes to the clinically observed pigmentation in smokers and non-smokers, and to calculate the size of melanosome complexes, 15 patients in the age 15–50 years were included (IV, VI). All of the eleven smokers and two of the non-smokers presented a clinically visible gingival melanin pigmentation. Another two non-smokers showed no clinical oral melanin pigmentation and served as controls. Relevant clinical data (drug consumption etc) was registered for all these patients.

Tissue biopsies

Gingival biopsies were taken in connection with periodontal surgery or as routine biopsies (I, IV, VI). In (II), biopsies were taken during the main investigation (Axéll 1976). The tissue samples in study IV and VI were selected as follows:

SMOKERS

1. CLINICALLY PIGMENTED AREA of the labial attached gingiva in the anterior part of the maxilla or mandible medial to canines. The biopsy included the interdental papilla and the whole attached gingiva down to the mucogingival border at the areolar mucosa.
2. CLINICALLY NON-PIGMENTED AREA of the posterior gingiva in the maxilla or mandible, corresponding to the gingival tissue excised in the pigmented area (see above), but taken from the buccal aspect of the attached gingiva and gingival papilla medial to one of the first molars.

NON-SMOKERS

No clinical pigmentation could be recognized in this group. Pieces of gingiva were excised, corresponding to those taken from the anterior region of the mandible in smokers.

TISSUE PREPARATION

Light microscopy (I, III, IV, V & VI)

In (I), all 18 pigmented out of the 121 examined individuals were biopsied. These biopsies as well as those from all 15 patients in study IV & VI, and the amphibian tissue (III, V), were fixed in 10% formaline, dehydrated, embedded in paraffin and stained with hematoxylin-eosin. Additional tissue (I) was stained with silver nitrate (Masson-Fontana) to show melanin, and incubated with DOPA (Bloch 1927) to show DOPA-positive melanocytes.

Electron microscopy (IV, VI)

Human and amphibian tissue was immediately fixed in chilled (+4°C) 2.0 % glutar-aldehyde for 2.5 hours, and then transported to cold cacodylate buffer. Thin slices of tissue were cut with razor blades and post-fixed in 1 % osmium for 1 hour. The tissue slices were embedded in Epon (Luft 1961). From each biopsy specimen an average of 10 tissue slices were embedded and thick sectioned. One micron sections were stained with toluidine blue for light microscopical orientation.

Ultrastructural studies

The most heavily pigmented areas of the epithelium were selected by light microscopy for ultrathin sectioning. In case of no pigment, as in sections from clinically non-pigmented non-smokers, areas were selected which topographically corresponded to those selected in smokers. Ultrathin sections were cut with an LKB Ultratome, collected on copper grids and contrasted with uranyl acetate-lead citrate. The sections were examined in a Zeiss EM 10A electron microscope. Of all the embedded tissues approximately 50 % of clinically pigmented tissue from smokers, 35 % of clinically non-pigmented tissue from smokers, and 60–75 % of tissue from non-smokers were thin sectioned and examined in the EM.

Low magnification micrographs were taken from all areas of the epithelium where electron dense pigment granules and/or clear cells could be identified. Micrographs with higher magnification were taken of keratinocytes and melanocytes. Melanosomes at different stages of

maturation were identified according to the morphologic criteria established by Fitzpatrick et al. (1969). The number of basal keratinocytes containing melanin was calculated in order to determine the ultrastructural degree of melanin pigmentation, and to correlate this to the clinically visible state of pigmentation (IV). Melanosome complexes (MC) within keratinocytes were selectively studied (VI). The size of 770 MC, taken as the largest diameter from micrographs with magnification of 8000 to 25 000 X, was measured to the nearest of 0.1 mm under a magnifying glass.

EXPERIMENTAL STUDIES (III, V)

In order to study amphibian skin in the light microscope and in the spectrophotometer during nearly identical conditions, a new specimen holder was constructed (III).

By spectrophotometry it was possible to study the darkening and lightening of toad and frog skin i.e. melanocyte activity changes during stimulation by specific agents (V). The influences of theophylline, α -MSH and isoproterenol were compared to that of nicotine. Skins kept in frog Ringer solution served as controls. A Zeiss spectralphotometer PM6 was used at a wavelength of 530 nm (Bitensky & Burstein 1965).

The melanocyte activation by isoproterenol was compared to that of nicotine during 30–90 min by in vitro light microscopy. After deactivation in frog Ringer solution and incubation in 0.1 mM propranolol during 30 min a reactivation test was initiated with isoproterenol or nicotine. The reactions were registered photographically (V).

RESULTS

STUDY I

Between 12.9 and 14.9 % of the individuals showed gingival pigmentation, which corresponded to 25 % and 29 % of the tobacco smokers. The light microscopic study showed strongly silver-stained keratinocytes, and DOPA-positive and silver stained dendritic cells in the basal epithelium. In the electron microscope the pigment was identified as melanosomes, which were present in dendritic melanocytes as well as in keratinocytes. Fifty percent of the individuals were pigmented only in the central part of the labial interdental papillae, while in individuals with the strongest pigmentation it covered most of the interdental papillary and attached gingival area medial to canines. The pigmentation was most common in the mandible ($p < 0.001$) and medial to canines ($p < 0.001$). In the strongest pigmented individuals a concomitant pigmentation of the maxillary labial gingiva was often observed. All pigmented individuals were smokers.

The pigmented smokers consumed more cigarettes than non-pigmented smokers ($0.01 > p > 0.001$). Pigmented males smoked a larger number of cigarettes as compared to pigmented females ($0.05 > p > 0.01$). It was noted that more than 50 % of pigmented individuals had a blue or grey iris and an ash-colored or blond hair. Thus, no correlation was found between a dark complexion and gingival melanin pigmentation.

STUDY II

In the general population the prevalence of gingival pigmentation was 9.9 %. No significant sex difference was noted. The pigmentation was most common in the age of 25–34 years (15.9 %). The most common location was in the labial attached gingiva in the mandible and in

falling frequency in buccal mucosa, buccal gingiva in premolar and molar areas, anterior buccal mucosa and in the labial mucosa. The correlation between pigmentation and the use of tobacco observed in study I was confirmed. In non-tobacco users only 3 % were pigmented as compared to 21.9 % among cigarette smokers. In individuals smoking more than 15 cig/day 30.9 % were pigmented. Among pipe smokers 16.8 % were pigmented and among snuff dippers 4.7 %.

A very strong correlation was found between cigarette consumption and the number of pigmented individuals grouped according to the number of cigarettes smoked each day ($r = 0.98$; $0.01 > p > 0.001$). This corresponds to an index of determination of 0.97, showing tobacco smoking statistically to be responsible for 97 % of the pigmentation observed.

Among females who smoked up to 15 cig/day there were significantly more pigmented individuals as compared to males with the same consumption ($p < 0.001$). This confirms the sex difference found in study I. The number of smoking years was also found to influence the prevalence of pigmentation. Already one year after start of smoking a significant rise of pigmented individuals from 3.0 % to 14.6 % was noted. Thereafter the prevalence was higher the longer the duration of the habit. Also the reverse was seen, as the number of pigmented individuals was lowering after cessation of smoking. After a 3 year stop about the same number of pigmented individuals was found as among non-smokers i.e. 3 %.

A certain specificity as to location within different mucosal regions was noted in some tobacco habits. Pipe-smokers more often showed buccal mucosal pigmentation compared to men smoking up to 15 cig/day or to snuff dippers. Snuff dippers were more often pigmented in the labial attached gingiva of the maxilla and in the buccal mucosa as compared to men not using tobacco. No clinical pigmentation was noted in the vestibular region where the snuff was placed.

STUDY IV

In the basal cells of the human gingival epithelium yellow to brown pigments were seen in the light microscope, and concentrated to the tips of the rete pegs. Large amounts of pigment were noted within clinically pigmented regions, while the amount was lower in clinically non-pigmented areas from smokers, and was rarely found in non-pigmented non-smokers.

Ultrastructurally no electron dense material other than melanosomes was found corresponding to the clinically and light microscopically observed pigmentation. The melanosomes were situated in melanocytes and keratinocytes, but melanosome-like granules were also observed sparsely in macrophagic and fibroblastic cells within the connective tissue. Melanocytes were observed in all biopsies and were present in the two–three lowermost basal epithelial cell layers. They were identified by their light cytoplasm, the absence of desmosomes, and their content of melanosomes in different degrees of maturation. In clinically pigmented tissue from smokers and non-smokers and in clinically non-pigmented tissue from smokers the melanocytes had an identical morphology. The nucleus was slightly indented and melanosomes at stage II–IV of maturation were seen in the cytoplasm, especially in the well developed dendrites. In the elongated dendrites bundles of fine filaments were seen running parallel to the cell membrane.

In pigmented tissue from smokers 83 % of basal keratinocytes contained melanosomes mainly as melanosome complexes, and often grouped around the nucleus. Very few or no melanosomes were observed in suprabasal cell layers. A similar observation was made in non-pigmented tissue from molar regions in smokers, where 45 % of keratinocytes contained melanosomes, as well as in clinically pigmented tissue from non-smokers where 64 % of the keratinocytes showed melanosomes. In tissue from non-pigmented non-smokers only 4 % of the keratinocytes contained melanosomes.

STUDY VI

The cross cut diameter of melanosome complexes (MC) was measured in keratinocytes of clinically pigmented individuals. Very few or no melanin was found in clinically non-pigmented participants. The diameter of 770 MC was found to be between 0.51 and 0.63 μm , which is larger than those previously reported from clinically non-pigmented Caucasian gingiva. In 95 % of measured MC the diameter was less than 1.0 μm but a number of larger, so called large melanosome complexes (LMC) were also found.

STUDY III

Compared to earlier described devices (Shizume et al. 1954, Bitensky & Burstein 1965) the present device permitted the skin specimens to be kept mounted and submerged in test solution during both spectrophotometry and light microscopy, and both sides of the specimen could be studied. Relatively small pieces of skin, 0.5 cm^2 , could be used, which compared to earlier devices lowered the number of animals necessary for the tests. Also the possibility of keeping the test solution under a seal during the tests was an advantage, and did not hinder the exchange of test solution or introduction of a fixative.

STUDY V

Light microscopically epidermal and dermal melanocytes were identified in the skin of toad and frog. The epidermal melanocytes were few and small compared to dermal melanocytes. The latter were flat and seemed to spread their dendrites mainly horizontally. Connective tissue melanocytes were seen at two different tissue levels i.e. close to the epidermal basement membrane (high level dermal melanocytes), and deep in dermis close to the split skin surface (low level dermal melanocytes). High level cells were often seen in contact with iridophores and xanthophores.

Spectrophotometric studies showed a very clear increase of light absorption in toad skin influenced by theophylline, MSH and isoproterenol, indicating an activation of melanocytes with melanosome distribution out in dendrites, thus making the skins more pigmented. Also 10 and 15 mM nicotine solutions resulted in an increased light absorption, but to a somewhat lower degree. The nicotine activation was reversible after washing with frog Ringer solution.

In vitro light microscopy showed high- and low level dermal melanocytes in toad and frog to be activated by α -MSH, theophylline, isoproterenol and 10 and 15 mM nicotine. The activation was also possible to see by the naked eye, as a darkening of the skin. Frog iridophores showed dendrites when immersed in control solution, but were inactivated to a non-dendritic shape by nicotine, quite contrary to melanocytes (III). The light microscopic reactions were confirmed ultrastructurally (V). Low level melanocytes were more easily activated than high level cells, and frog melanocytes more easily than toad cells. To study if nicotine activated the melanocytes via adrenergic β -receptors, isoproterenol served as control. The melanocyte activation with isoproterenol was completely blocked by the β -receptor blocker propranolol. However skins incubated in propranolol, and stimulated with nicotine, showed the same degree of activation as prior to β -receptor blocking. Therefore nicotine stimulates dermal melanocytes in a non-toxic way and this stimulation is not mediated via catecholamines and β -receptors. Other receptor mechanisms must be involved.

DISCUSSION

EPIDEMIOLOGY

A very strong correlation was found between oral melanin pigmentation and tobacco smoking (I, II). The epidemiologic findings of pigmentation were confirmed histologically. In the population from central Sweden (II) 3.0 % among non-tobacco users were pigmented. This would correspond to 1.7 % of the total population. The factors causing pigmentation in non-smokers are not fully known, but the main part of this pigmentation is probably due to genetic influence, and partly to melanocyte stimulating agents in food and medical drugs. In the northern population only individuals born in Scandinavia were present (I) while in central Sweden (II) 1.0 % were immigrants from southern Europe. Of the latter the main part were Greeks among whom 16.0 % were pigmented, compared to 9.9 % in the total population. This low number of south Europeans, where a genetic oral pigmentation is supposed to be more common, was therefore not of a magnitude influencing essentially on the prevalence. General pathologic conditions such as Addison's disease, Peutz Jegher's syndrome, and other diseases where oral melanin pigmentation is a common clinical sign, are rare in Sweden, and are not influencing our prevalence figures.

Study I and II show oral melanin pigmentation to be present in 9.9 – 14.9 %. A larger number of pigmented individuals were found when more tobacco users were present. Tobacco smoking was found to be statistically responsible for 97 % of the observed pigmentation. Therefore the concept "smokers' melanosis" was introduced (I) making a distinction possible between a tobacco induced oral melanin pigmentation and a genetically, medically or a food-factor induced pigmentation.

The low prevalence of oral melanin pigmentation found in the Swe-

dish population is in agreement with the literature, showing the genetical influence in Caucasians to be low (Fry & Almeyda 1968, Eleutério 1969). In Germany (Özbayrak 1983) 1.3 % among 1552 individuals showed a pigmentation which could not be linked to any tobacco habit, (1.7 %, II) which corresponded to 2.9 % among non-smokers (3.0 %, II). Among smokers 18.7 % were pigmented (21.5 %, II). A prevalence of 9.9 % of oral melanin pigmentation (II), where smokers' melanosis is responsible for 6.8 %, makes melanin pigmentation one of the most common intra-oral lesions after leukoedema, recurrent aphtous and denture stomatitis (Axëll 1976).

No sex difference of prevalence was noted *in the total population*. However, a difference was found *among smokers*, showing females to be more often pigmented as compared to males (I, II). This was highly significant in the group smoking up to 15 cig/day ($p < 0.001$). The higher sensitivity of the female oral mucosa to tobacco smoke, seen as an elevated melanin production, is supposed to be due to the priming of melanocytes by female sex hormones. This theory is supported by the finding of an exaggerated skin melanin pigmentation around eyes and perioral regions after UV-irradiation in pregnant females, and in those taking the pill (Snell & Turner 1966, Resnik 1967, Sanchez et al. 1981). During pregnancy estrogen and progesterone levels are elevated (Hugoson 1971). Ovariectomy in females lowers the skin melanocyte activity, while medication with estrogen and to a certain extent with progesterone restores the skin pigmentation (Snell & Bischitz 1963). Tobacco smoking is however reported to elevate the liver metabolism of hormones, and thus to lower the plasma estrogen levels in females, as well as that of testosterone in beagle dogs exposed to tobacco smoke (Mittler et al. 1983, Baron 1984). The observed sex difference in skin melanin pigmentation is also significant in individuals medicating with polycyclic amines such as phenothiazines or phenytoin. Here the skin melanin content is significantly elevated particularly in females, both among Caucasians and Bantus (Robins 1972).

The regional differences between smokers' melanosis appearing in cigarette smoking, pipe smoking and snuff dipping show that tobacco

stimulates the melanocytes and keratinocytes locally. A local melanin pigmentation is also observed in lichen planus (Murti et al. 1979, Laskaria et al. 1981). In this lesion inflammation has been proposed to induce the melanin pigmentation. A similar observation is made in the clinically pigmented human gingiva, where the light microscopic appearance of silver-staining melanosomes has been positively correlated to inflammatory cells in the upper part of connective tissue (Patsakas et al. 1981). However, no distinction is made as to etiology of the melanin pigmentation studied. For instance a genetically pigmented gingiva is not supposed to be positively correlated to gingival inflammation. Also smokers' melanosis is known to be present in clinically healthy gingiva (I). Ultrastructurally, inflammatory cells were seen in the epithelium of pigmented individuals but to a very low extent (IV). In the gingiva of man several clinical signs indicate that tobacco smoking may lower the degree of inflammation. Tobacco smoking is associated with less bleeding, less gingival exudate and less redness of the gingival tissue, as compared to the gingiva in non-smokers with periodontitis (Hedin et al. 1981, Preber & Bergström 1985, Preber 1986). A chronic irritation through burning with cigarettes in the Bantu lip mucosa results in a depigmentation (van Wyk 1976). Thus, there seems not to be a direct relation between inflammation and melanocyte activation. However in injured tissue toxic products from dying cells are present, and may in agreement to our theory be possible stimulators of melanocytes, and may also bind to melanin.

Non-local factors are also known to influence a melanin pigmentation. The genetic factor causing a constitutional melanin pigmentation is the most well known of these. In skin and oral mucosa hormones may produce pigmentation for instance in Addison's disease. UV-irradiation to one ear of mouse also stimulates melanocytes in the shielded control ear (Rosdahl 1979). An endogenous melanocyte stimulator seems therefore to be produced by the irradiated tissue. Other endocrine factors possibly correlated to tobacco smoking and melanin pigmentation will be discussed later.

The conclusion is that the assumption in early 20th century of a

connection between tobacco smoking and oral pigmentation has now been confirmed, and the pigment is identified as melanin (I, II, IV). Further epidemiologic studies on smokers' melanosis have shown the present observations to be valid also in Danish and German populations (Larsen 1980, Özbayrak 1983). The recognition of smokers' melanosis has also contributed to the elucidation of oral pigmentation in a group of Japanese typographical workers. Lead was suspected to be the reason for an observed gingival pigmentation, but the pigmentation was found to consist of melanin, and to be caused by tobacco smoking (Araki et al. 1983).

The epidemiologic and ultrastructural studies (I, II, IV, VI) demonstrate that the clinical pigmentation in smokers was due to an increased production of melanin by activated melanocytes. Not only did the findings raise the question of the mechanism by which smoking causes the melanocyte activation. Also, the physiologic or functional significance of the smoke-induced tissue reaction seems like an intriguing problem to solve.

THE MELANOCYTE

The clinical appearance of a melanin pigmentation like smokers' melanosis is dependent on the amount of melanin transferred from the melanocyte to surrounding cells (IV). It is therefore of interest to compare the ultrastructural morphology of melanocytes and surrounding cells in other pigmentary conditions to that of smokers' melanosis. This could answer the question if smokers' melanosis is a pathologic lesion or an unspecific cell reaction.

MORPHOLOGY

The melanocyte derives from the neural crest and migrates towards dermis during the 10th week of foetal development. In cell cultures melanocytes are seen to move over 20 epithelial cells during 30 min. Usually the melanocyte is relatively stationary and is through dendrites

in connection with some 36 Malpighian cells, and one epithelial cell is in contact with several melanocytes (Cohen & Szabo 1968).

During a period of extensive research on pigmented skin, the functioning unit of melanocytes and keratinocytes was given the name EPIDERMAL MELANIN UNIT (Fitzpatrick & Breathnach 1963). However, studies on man, other mammals, birds and several other species have shown melanocytes and epithelial cells to interact also in extracutaneous regions. In man, melanocytes are for instance regularly found in the retinal, oral, pharyngeal and vaginal epithelium, and also in stria vascularis of the cochlea, and in planum semilunatum of the ampullæ in the inner ear (Schroeder 1969A, B, Mishima et al. 1978, Jones 1979, Lin & Zak 1982). Accordingly there is a need for a new name, and we therefore propose the concept EPITHELIAL MELANIN UNIT which will be applicable to all epithelium lined body surfaces.

The role of the melanocyte is to produce melanin, to store it on the premelanosomes, and to transfer the melanosomes to surrounding epithelial cells. In some species melanocytes are also present in the connective tissue, for instance in the mouse (Rosdahl 1979) and in amphibians where, in the latter, the movements of melanosomes mainly are restricted to a back and forth movement within melanocytes (Taylor & Bagnara 1972).

The dendritic cells of the epidermis were first reported by Paul Langerhans (1868). He seems to have recognized the dendrites of melanin-bearing melanocytes. Becker (1927) found DOPA-positive melanocytes in the basal epithelial layers of the oral mucosa, and most numerous in the tips of the rete pegs. In the Caucasian gingiva melanocytes were found in all individuals, though no clinically visible pigmentation was observed (Laidlaw & Cahn 1932, Berman 1940, Breijer & Lignac 1953). The number of DOPA-active melanocytes is largest on the top of the free gingiva and then rapidly decreases within the gingival pocket, and is not seen deeper down than 0.2 mm by the light microscope (Barker 1967). Ultrastructurally solitary melanocytes are seen to a depth of 1.4 mm (Squier & Waterhouse 1967, Schroeder 1969D). In the human gingiva 520 DOPA-active melanocytes have been observed per mm²

(range 195–1171) which corresponds to one melanocyte in every 15 keratinocytes (Barker 1967, Soames 1974). In skin the average number of melanocytes is 1155/mm² but the number varies considerably between individuals, between different skin regions and also depending on the staining method. In spite of this no significant difference in melanocyte numbers has been found between Caucasians and dark colored ethnic groups (Pinkus et al. 1957).

Gingival melanocytes were first studied ultrastructurally by Schroeder & Theilade (1966) and Thilander (1968). In the electron microscope a non-active melanocyte is characterized by a round or oval nucleus, non-melanized melanosomes in the cytoplasm, and short dendrites devoid of melanosomes. The Golgi apparatus and EPR are undeveloped (Schroeder 1969A). Microfilaments and solitary microtubules are situated close to the nucleus in epidermal melanocytes (Drzewiecki & Piltz-Drzewiecka 1979). In contrast, an active melanocyte shows an indented nucleus and a well developed Golgi region as well as EPR. Between 40–130 melanosomes in all stages of maturation are seen in the sectioned cell. The crosscut dendrites are slender and contain plenty of melanosomes (Hashimoto et al. 1966A, Schroeder 1969A).

The maturation of melanosomes in epidermal melanocytes has been ultrastructurally distinguished in four stages, I–IV (Fitzpatrick et al. 1969). Stage I–III, also called premelanosomes, are the unmaturing melanosomes consisting of a protein matrix without any melanin deposition (stage I) or with the internal structure visible (stage II) or partly filled with melanin (stage III). Stage I melanosomes are difficult to distinguish from other lysosome-like structures (Nakagawa et al. 1984). Stage IV is the mature melanin granule. These stages are also found in the melanocytes of the oral mucosa (Haim 1964, Squier & Waterhouse 1967, Schroeder 1969A). This is in accordance with the present studies, where melanosomes in stages I and II were characteristic findings in the clinically non-pigmented non-smokers, while in pigmented individuals stages III and IV predominated (IV).

The melanin-producing melanocyte will stain with DOPA (dihydroxy phenyl alanine) for light microscopy. DOPA, will serve as a sub-

strate for melanin in melanocytes which are active and contain tyrosinase (Bloch 1927). Melanin stains with silver nitrate (Masson-Fontana and others) and is thus seen in melanocytes as well as in keratinocytes. Melanocytes are DOPA-positive in smokers' melanosis, and the produced melanin stains well with silver (I). These staining methods have also been found valid in ultrastructural studies. DOPA-staining shows the tyrosinase-active regions in Golgi vesicles, cisternæ and melanosomes of melanocytes (Yamamoto & Takeuchi 1981), and silver stains the melanized melanosomes (Fitzpatrick et al. 1969, Sirsat & Deo 1981).

MELANOSOME TRANSPORT

The transport mechanism of the melanosomes in the melanocyte has been studied in several species for instance in fish scales, amphibian and human skin. In the resting cell melanosomes make a Brownian shuttling-like movement, moving 2μ in one min. After hormone activation a collective movement of all melanosomes is observed directed towards dendrites. Melanosomes now move with a speed of $2-6\mu$ per second (Green 1968). Ultrastructural studies have shown the movement of granules to be due to the conversion of microtubules into microfilaments, and the latter are found in dendrites intermingled with melanosomes. This granule movement is enhanced by colchicine, which is known to disintegrate microtubules to microfilaments (Moellmann et al. 1973). When microtubules are rebuilt the melanosomes in fish and amphibians return to nuclear regions. This is experimentally possible to hinder by colchicine and vinblastine, agents preventing microtubules to redevelop (Moellmann & McGuire 1975). Similar observations are made in guinea pigs where inactive melanocytes mainly contain microtubules (Moellmann et al. 1973).

Ultrastructural studies of human epidermal melanocytes not influenced by UV-irradiation, show $100\text{ \AA}$ microfilaments to be present in parallel bundles around the nucleus. Microtubules are few but seen near the nucleus and in dendrites. Already at 30 min after UV-irradiation an aggregation of microfilaments and melanosomes is seen in the central

part of dendrites, while microtubules are present in their periphery. These are morphologic features consistent with those found in smokers' melanosis (IV). The gingival pigmentation has also other prominent morphological similarities with the locally induced, and essentially non-endocrine melanin pigmentation obtained by UV-light. The slow developing and long lasting pigmentation produced by UV-B light (290–320 nm) shows a large number of melanin-bearing keratinocytes, and long slender melanosome-filled dendrites of the activated melanocyte. In the tip of dendrites microfilaments form a mesh containing melanosomes in different stages of maturation (Jimbow et al. 1973, Jimbow & Fitzpatrick 1975). The same findings are reported in smokers' melanosis (IV). The observed microtubules and filaments are parts of the skeleton of the cell cytoplasm, called the microtrabecular lattice.

Also in the present amphibian studies on frog and toad (III, V) a centrifugal movement of melanosomes from the nuclear region out into dendrites was seen in the stimulated melanocyte. After washing of the tissue, a centripetal movement of melanosomes towards the nucleus was seen in the inactivated cell. This corresponds well with the findings in the literature (McGuire 1972). The light microscopic morphology of the initial melanocyte activation was nearly identical to that of a following reactivation (V). The finding in nicotine-treated toad and frog skin melanocytes indicates that microfilaments are developed, permitting a normal movement of melanosomes out into dendrites. This has to be followed by a rebuilding of microtubules after the washing out of nicotine, because an undisturbed centripetal movement of melanosomes was observed.

TRANSFER OF MELANOSOMES TO KERATINOCYTES

The transfer of melanosomes from the melanocyte to the epithelial cell is an active process involving both cells. This is performed through a cytophagocytosis of the melanosome-containing dendrite tip by the keratinocyte, transfer of granules from the melanocyte to the keratinocyte through cytoplasmic bridges or by the phagocytosis of free extra-

cellular melanosomes (Mottaz & Zelickson 1967, Okazaki et al. 1976, Jimbow 1979). When the tip of the dendrite is pinched off, the group of melanosomes move towards the nucleus of the keratinocyte. This group of granules is called a melanosome complex (MC) and contains between 5–20 melanosomes in cross cut Caucasian skin keratinocytes (Mottaz et al. 1971, Hu 1979). Also in Asians melanosomes are in complexes but are grouped closer than in Caucasians. In dark skinned Negroes the melanosomes are dispersed singly in the cytoplasm and are larger (Szabó et al. 1969). The size of the granule has morphologically and experimentally been shown to determine if melanosomes are to be grouped as MC or to appear dispersed as single granules (Wolff & Konrad 1972, Wolff et al. 1974). No absolute correlation has been found between skin color and melanosome distribution within keratinocytes, because also in dark skinned Negroes MC are sometimes seen (Konrad & Wolff 1973, Olson et al. 1973). Experimentally small granules grouped as MC are replaced by larger, single melanosomes in Caucasian skin after UV- and Psoralen treatment (Toda et al. 1972). The critical granule size for the formation of MC vs dispersing single melanosomes is found to be 0.50–0.80 μm (Wolff 1973, Olson et al. 1973). We did not study the size of individual melanosomes in keratinocytes, but reported both MC and single melanosomes in the same tissue specimen (IV).

The prominent finding was that of larger MC in smokers and pigmented non-smokers as compared to clinically non-pigmented individuals, which is in accordance with findings in UV-irradiated skin. Here not only melanosomes but also MC become larger after UV-irradiation. MC in Caucasian skin normally contain 2–3 melanosomes prior to UV-irradiation but show 5, 10 or more granules after UV-activation (Hori et al. 1968, Wolff & Schreiner 1971).

MC in the size of 1.0 μm or larger, so called large melanosome complexes (LMC) were also found (VI), and are not earlier reported from the human oral mucosa. They are however seen in epidermal *melanocytes* from patients with malignant melanomas, nevi and lentigo simplex, but also in normal skin. Here LMC could reach 3–10 μm (Hirone & Eryu 1978, Drzewiecki 1979, Drzewiecki & Piltz-Drzewiecka 1979,

Horikoshi et al. 1982). They can be produced experimentally by tape stripping of skin, and are supposed to be due to a disturbance in the normal cell function (Hirone & Eryu 1978, Drzewiecki 1979). Edema between cells has been proposed to inhibit the transfer of melanosomes from melanocytes to keratinocytes (Mottaz et al. 1971). LMC are hitherto only reported *in keratinocytes* in connection with nevus spilus and multiple lentigines syndrome. Here up to one hundred melanosomes were seen in the complex (Hu 1979). This is similar to the finding of LMC reaching $3.2\text{ }\mu\text{m}$ and containing more than one hundred melanosomes in the pigmented gingiva (VI).

In the present studies (IV, VI) LMC were mainly found *in keratinocytes*. Therefore no faulty transfer of melanosomes from melanocytes to keratinocytes was present. In suprabasal cell layers a normal melanosome degradation was noted. Few or no melanosomes were seen in the upper epithelium, and the clinical pigmentation is therefore due to a larger number of melanosomes only in basal cells. As LMC are seen in normal skin melanocytes (Drzewiecki 1979) which may be influenced by UV-light, and because no ultrastructurally evident pathologic findings are hitherto observed in the epithelial melanin unit of the oral mucosa in smokers' melanosis, the formation of LMC may be the consequence of an increased number of transferred melanosomes due to a strong stimulation of melanocytes and keratinocytes. These findings raise the question of the physiologic function of the melanin hyperproduction in smokers' melanosis.

FUNCTION OF MELANIN

WHAT IS MELANIN?

The concept of "melanin" was first used by J.J. Berzelius in the beginning of the 19th century, to denote a dark, non-soluble pigment (Riley 1980 review). Early studies showed mushrooms and herbs to produce melanin. If the cut surface of a potato, apple or banana is exposed to oxygen it becomes black (Pawelek & Körner 1982). Here melanin is produced by oxidizing nitrogen-free phenolic substrates to qui-

none, which is then oxidized to melanin (Edelstein 1971 review).

Three different types of melanin are found in mammals – eumelanin, pheomelanin and trichochromes. Eumelanin is characterized by its indole content, pheomelanin by a high sulphur content, and both may be produced by the same melanocyte (Sakurai et al. 1975). Trichochromes are characterized by their low molecular weight. Eumelanin gives the brown or black color to human skin and hair, while pheomelanins are present in hair from red-haired individuals, and in the coat of the rabbit, sheep and goat. Also trichochromes are present in blond and red human hair, and are found in the urine from these individuals, but also in the yellow, red and violet feathers of birds (Prota 1980).

The formation of melanin is mediated by tyrosinase. This enzyme hydroxylates the amino acid tyrosine to DOPA (Bloch 1927) which is dehydrogenated to DOPA-quinone. Through oxidation DOPA-quinone is converted to dihydroxy-indole which with or without the influence from tyrosinase is oxidized to the monomere indole-5,6-quinone. This monomere forms together with similar molecules a polymer, and thus forms melanin (Mason 1959, Fitzpatrick & Kukita 1959, Riley 1980). Besides this type of reaction melanin may be formed by dopamine, tryptophan and through oxidation of epinephrine, the latter in a defective liver (Edelstein 1971). In certain circumstances peroxidase may replace tyrosinase as the enzyme hydroxylating tyrosine (Okun et al. 1973).

MELANIN AS A FREE STABLE RADICAL

A free radical is a chemical substance with an unpaired number of electrons and because of that it is very reactive and unstable. Melanin is a stable free radical, and has the potential to bind a large number of substances through its very reactive quinones (Prota 1980). In tissue influenced by UV-light, the photons dissociate covalent couplings, and produce single, unpaired free electrons, i.e. free radicals (Riley 1980). Melanin has the capacity to bind the free radicals produced by the photons in the tissue, and thus prevent tissue damage. This is achieved by

melanin serving as an electron transfer agent in several reduction-oxidation systems (Gan et al. 1976). After UV-irradiation of skin the amount of free radicals is 50 % higher in the less melanized skin of Caucasians as compared to Negro skin (Pathak & Stratton 1969).

Free radicals are also found in the gas phase of tobacco smoke, in tobacco tar and in cold tobacco. Among these are the alkoxyl radical, radicals bound to organic molecules in the smoke, benzenes and semi-quinone-radicals in the tar (Pryor et al. 1985). The free radicals in tobacco are supposed to bind to electron-accepting systems in the human oral and respiratory mucosa. Important biochemical redox-systems may be disturbed (Rowlands et al. 1968). Our theory is that an increased amount of melanin in the oral mucosa could perhaps act by binding the free radicals entering the tissue from tobacco, and in this way prevent tissue damage.

In the pig gingiva free oxygen radicals depolymerize hyaluronic acid and proteoglycans, two of the most important extracellular macromolecules in the connective tissue (Bartold et al. 1984). In vitro studies using bovine eye melanin, show melanin to compete effectively with superoxide dismutase in the conversion of superoxide-radicals to H_2O_2 and unharmed O_2 (Korytowski et al. 1985). Our hypothesis is that the lower degree of gingival inflammation in smokers with periodontitis compared to non-smokers, partly is due to the larger amount of melanin in the gingiva of smokers (see also page 12). The melanin may lower the amount of free oxygen radicals produced by the inflammation in the tissue, and thus serve as a complement to superoxide dismutase. However, melanin has also the capacity to bind potentially harmful polycyclic amines, entering the oral mucosa from the tobacco. This is another interesting task for the melanin present in smokers' melanosis.

MELANIN AND POLYCYCLIC AMINES

In patients taking chlorpromazine or chloroquine the drugs were observed to be disposed in the uveal melanin, and were associated with

a deterioration of sight. The medicals could be dissociated from this binding without any change of the chemical structure (Potts 1964). The binding to melanin seemed to be specific and applicable to several substances foreign to the organism. Melanin is known to bind them for a very long time, sometimes for years (Potts 1964, Blois 1972). The binding takes place in several organs all shown to contain melanin. The chemicals bound are all of a polycyclic configuration, and consist of two or more cycles where nitrogen is present in the cycles or in side chains i.e. polycyclic amines (Lindquist & Ullberg 1975). Among these agents are kanamycin, tetracycline, streptomycin, chloroquine, quinine, phenothiazines, alkaloids, different color agents, benzopyrenes and also nicotine. In animal experiments where nicotine and polycyclic amines are injected in pigmented animals, a specific and long time binding takes place in melanin-bearing regions of the stria vascularis of the cochlea, planum semilunatum of ampullæ, substantia nigra and locus coeruleus of the brain (Lindquist 1973, Szűts et al. 1978).

The polycyclic amines seem first to enhance the melanin production, followed by a cell degeneration with the spreading of melanin to surrounding tissue, and to the reticulo-endothelial system. Damage to the organs resulting in impairment of sight, balance, hearing, and depigmentation of neurons in substantia nigra giving Parkinson-like symptoms, is noted. This phenomenon is explained by a sudden release of high concentrations of the chemical, and the clinically and ultrastructurally observed destruction may be the result of damage to the nutrition of the organ (Lindquist 1973, Wästerström 1984). We found no evidence that such morphologic changes also occurred in the epithelium of smokers' melanosis (IV, VI), and this can perhaps be explained by the rapid cell cycle of melanin-containing keratinocytes in the oral mucosa. The cells are expelled from the epithelial surface prior to a release of the bound toxins.

The available data clearly indicate that nicotine from tobacco smoke may bind to melanin. An ultrastructural comparison of the clinical pigmentation obtained after the intake of drugs with a polycyclic nature, to that of smokers' melanosis, could give information on how ni-

cotine, benzopyrene, and other polycyclic amines in tobacco influence the oral mucosa.

MEDICAL DRUGS AND PIGMENTATION

SKIN PIGMENTATION

Clinically visible grey to blue-grey skin pigmentations have been reported after the use of phenothiazines such as chlorpromazine and the tetracyclines Minocycline, Methacycline and Doxycycline (Greiner & Berry 1964, Kuske & Krebs 1964, Forrest et al. 1966, Robins 1972, Möller & Rausing 1980). The histopathologic findings are similar in pigmentations caused by chlorpromazines and tetracyclines. Abundant argentaffin granules are seen in the connective tissue especially close to vessels. These granules were thought to be melanin, but ultrastructural studies show two types of pigment, melanosomes and dense, structureless granules. Both types are often found together in the same secondary lysosome within macrophages, fibroblasts, and endothelial cells, and are therefore easy to distinguish morphologically. Light microscopically staining with Prussian blue shows some granules with iron content (McGræ & Zelickson 1980). This was confirmed by electron X-ray microanalysis (Sato et al. 1981). In the epithelium a large number of ordinary melanosomes are also seen within keratinocytes (Hashimoto et al. 1966B). The conclusion was that melanosome production is stimulated and that the dense granule type in the connective tissue consists of drug metabolites (Zelickson 1965).

The mechanism of melanocyte stimulation by chlorpromazine is not fully known. Amphibian studies have shown an elevated MSH production and skin darkening in frog after stimulation by several phenothiazines. The pigmentation did not develop in hypophysectomized animals (Scott & Nading 1961). However, this frog skin pigmentation is not reversible, which would be the case if a true MSH-activation of melanocyte receptors was at hand. Therefore it seems possible that chlorpromazine very strongly binds to receptors on the melanocyte surface or causes injuries to the microtubules (Irwin 1972). In the present

studies (III, V) nicotine activated frog skin melanocytes via non β -adrenergic receptors. This skin pigmentation was fully reversible in contrast to the findings in the cited amphibian chlorpromazine studies.

DRUGS AND THE ORAL MUCOSA

Phenytines and especially antimalarials are known to produce oral pigmentations. Triquin and Camoquin are used also against rheumatoid arthritis and other connective tissue diseases. They may give blue-grey pigmentations located in the hard or soft palate, buccal- and lip mucosa or in the attached gingiva. The free gingival margin is not clinically pigmented, which is similar to smokers' melanosis (I). The absence of a clinical melanin pigmentation in this region contrasts to the very dense number of DOPA-positive melanocytes found in the gingival margin (Barker 1967). The gingival margin is the site of pigmentation due to the deposition of heavy metals such as gold, bismuth or mercury (ten Bruggenkate et al. 1975, Lockhart 1981). In Triquin and Camoquin induced pigmentation a strong perivascular pigmentation which bleaches with hydrogenperoxide is seen in the connective tissue, pointing to hemosiderin. In the epithelium silver stained granules point to melanin (Zachariæ 1963, Brynolf 1970, Giansanti et al. 1971).

The ultrastructure of smokers' melanosis has some similarities with the above medically induced pigmentation of skin and oral mucosa — the melanosome production is stimulated in the epithelium, and normal melanosomes are also found in the adjacent connective tissue. The finding of melanosomes in the connective tissue is however earlier reported in normal clinically non-pigmented Caucasians (Schroeder 1969C). In the present studies (VI) they are found together with larger electron-dense round granules within the cytoplasm of macrophages and fibroblasts. Our theory is that these granules may be metabolites from tobacco or of other origin. However, the findings in smokers' melanosis also contrast to those in pigmentations caused by medicals. In the present study the dermal pigment is clearly insignificant compared to the ambient melanin pigmentation found in the epithelium. In the

medically induced pigmentation the main part of the granules are reported to be situated in the dermis.

The drug-induced pigmentation in skin and oral mucosa appears a long time after the drug intake. In 4 months to 4 years of use chloroquines give a visible oral pigmentation. The lesions also take a very long time to disappear. A tetracycline pigmentation in the skin was still present after 5 years, and the oral pigmentation by chloroquine still after 1 year (Möller & Rausing 1980, Manor et al. 1981). In smokers' melanosis 6 months up to 3 years had to pass after cessation of smoking, until a drop and normalization in the number of pigmented individuals was noticed (II).

Although tissue of former smokers was not studied in the present investigation, the number of electron-dense granules in the connective tissue of smokers was discrete and could not explain the prolonged pigmentation in ex-smokers (IV). Therefore, as the dominant granule found was melanosomes in the basal epithelium, the probable cause for the remaining pigmentation in ex-smokers is a lasting melanocyte stimulation, similar to that seen after UV-B stimulation of skin (Toda et al. 1972).

MELANOCYTE REGULATORY MECHANISMS

Different mechanisms exist by which melanocytes can be stimulated. Some of these are relevant to discuss, in order to further understand how the melanocyte is activated in smokers' melanosis. Among the different chemicals in tobacco, where benzo(α)pyrene is known to have a melanocyte-stimulating capacity, nicotine was chosen because of its documented ability to stimulate catecholamine- and cAMP production, and because of the observed pigmentation in snuff dippers (II). How nicotine influences melanocytes is not earlier reported in the literature. The use of amphibian skin (III, V) is a well known in vitro model, by which the basic functions of the melanocyte and its receptor mechanisms have been studied during the influence of several important chemical agents and hormones.

AMPHIBIAN MELANOCYTES

In toad and frog, as in several other amphibians, specific cells called chromatophores, are found in epithelium and connective tissue, giving the color of the skin and permitting the well known fast color-changes in these species. Melanocytes belong to the chromatophores and are found in the epithelium and connective tissue. Epithelial melanocytes are small, often present in patches, comparatively inactive, disperse melanin to surrounding epithelial cells, and give the basic color to the skin.

In the connective tissue three different chromatophores are present forming a dermal chromatophore unit (Bagnara et al. 1968). They are melanocytes, iridophores and xanthophores, the latter called erythrophores when red. The dermal melanocytes are seen close to the basal lamina of the epithelium and in the deep connective tissue. They take part in the fast color-changes of the animal, and are therefore large, often horizontally oriented with radially directed dendrites covering a large skin surface. Within the dendrites matured round to oval melanosomes move back and forth in a specific order (Taylor & Bagnara 1972). When the amount of hypophyseal hormone in the blood is low the activity of the melanocytes is low. Melanosomes in the melanocytes are then concentrated around the nucleus, leaving the dendrites empty, light microscopically non-visible, and the skin looks pale to the naked eye. A high hormone production, makes melanosomes to stream out into dendrites, giving a large dark skin-area. In the present studies these morphologic alterations were confirmed by the light microscopic and ultrastructural observations (III, V).

HORMONES AND THE MELANOGENESIS

MSH (melanocyte stimulating hormone) and ACTH (adreno corticotropic hormone) are produced in the hypophysis (Doerr-Schott 1976). These two hormones show receptor stimulating similarities which may depend on a common amino acid sequence found in MSH

and ACTH (Snell & Kulovich 1969, Bagnara & Hadley 1973 review). MSH acts similarly on skins from amphibians and mammals, including man. After stimulation with α -MSH, a clinically and histologically visible pigmentation appears in amphibians (V), and also in mice, where the DOPA-positive melanocytes become more numerous (Snell & Kulovich 1969, Hirobe & Takeuchi 1977). If guinea pig epidermis in tissue culture is stimulated with α -MSH, the melanocytes will be activated starting a melanosome production, transport of melanosomes into dendrites, and a transfer of melanosomes to keratinocytes (Wikswa 1973). Injection with α -MSH in man will produce a visible melanin pigmentation in epidermis (Lerner et al. 1966).

A general skin pigmentation in man is also a characteristic finding in the ectopic ACTH-syndrome, which is caused by malignant neoplasms in the lung, pancreas, liver or thymus. These tumors have a local production of ACTH, α - and β -MSH (Daughaday 1974). The skin pigmentation has been observed to decline when the tumor is removed (Parker 1974). Also melanin pigmentation of the palatal and buccal mucosa has been observed in smokers with similar neoplasms (Merchant 1973, Merchant et al. 1976). Melanin pigmentation in the soft palate is rare in a Caucasian population (0.6 % of all pigmented regions observed, II). The buccal and palatal mucosa is however frequently pigmented in Inuits (Jakobsen 1968). This points to the need of including charting of the oral melanin pigmentation found in smokers and non-smokers among different ethnic groups in epidemiologic investigations. An unusually located melanin pigmentation calls for the need of a general medical examination of the patient.

Cortisol and cortisone both have an inhibiting effect on the hypothyseal hormone production. This control is lost when the adrenal cortex is destroyed in Addison's disease, with loss of feed-back control of β -MSH and ACTH production. Frog skin melanocytes are inactivated by cortisol and cortisone. In man tobacco smoking elevates the cortisol levels in plasma (Wilkins et al. 1982). By stimulating the release of cortisol tobacco possibly reduces the production of MSH and ACTH, and in this way decreases the endocrine melanocyte stimulation. How-

ever, in cultivated melanoma cells corticosterone stimulates to higher cAMP and tyrosinase-levels (Abramowitz & Chavin 1979, Edwards et al. 1981).

In man and amphibians skin melanocytes are regulated via an endogenous stimulation by hormones and catecholamines and not via nerves (Lerner et al. 1966, Fujii & Novales 1972). In amphibians three different receptor systems have been identified pharmacologically on the cell membrane (McGuire 1972). Besides the common MSH-ACTH receptor, an α - and a β -adrenergic receptor is present. Activation via the MSH-ACTH or the β -receptor stimulates the melanosomes to stream out into dendrites (Bagnara et al. 1968, Abe et al. 1969). The β -receptor is for instance stimulated by isoproterenol, and this reaction is blocked by the β -receptor blocker propranolol (McGuire & Möller 1966). An α -receptor activation by norepinephrine will move the melanosomes in a centripetal direction towards the centre of the cell. These melanocyte reactions on MSH and isoproterenol were also demonstrated on *Rana arvalis* and *Bufo bufo* (III, V).

It is well established that 3',5' adenosine monophosphate (cyclic AMP, cAMP) has a wide distribution in living organisms and has many different regulatory functions in the cell (Robison et al. 1968). In the melanocyte it has been confirmed that cAMP acts as a second messenger in mediating the effect of MSH, ACTH and β -receptor stimulation. Phosphodiesterases inactivate melanocytes and lighten skin by metabolizing cAMP to inactive non-cyclic AMP, and acetylcholine seems to act in a similar way (Moellmann et al. 1974). High cAMP levels are retained by phosphodiesterase inhibitors i.e. methylxantines such as theophylline and caffeine which in this way darken frog skin through melanocyte activation and aggregation of iridophore platelets (Bagnara & Hadley 1973). Also in the present study (V) theophylline activated amphibian melanocytes to skin darkening. Nicotine influenced the skin in a similar way by both activating melanocytes and by aggregating iridophore platelets (III).

As tobacco smoking positively influences on cyclic nucleotides in man only when nicotine is present (Takahashi et al. 1977), nicotine

seems to be the most prominent cAMP stimulating factor in tobacco. In study V the hypothesis was that nicotine through catecholamine stimulation may activate the melanocyte and darken the skin via the β -receptor. By comparing the melanocyte stimulation of nicotine to that of the well known β -receptor stimulator isoproterenol, and by blocking with propranolol, it was shown that nicotine does not activate or darken skin via β -receptors (V). Further studies are needed to reveal if nicotine acts via other receptors or through phosphodiesterase inhibition. We suggest the possibility of a hitherto unknown "nicotine-like" receptor in amphibian skin melanocytes, in line with recent findings in the central nervous system (Abood et al. 1980, Nordberg & Winblad 1981).

IS SMOKERS' MELANOSIS A PATHOLOGIC LESION, POTENTIALLY DELETERIOUS TO THE ORGANISM?

Malignant melanoma occurs in human skin but also in the oral mucosa. However, in contrast to skin manifestations, oral melanomas are rare. About one new case a year is registered in Sweden (Anneroth et al. 1973). Because of the poor prognosis of this lesion the general rule has been to excise all localized dark pigmentations in the oral mucosa, and to have them histologically examined. This advice is partly due to the impossibility of always making a correct diagnosis clinically on oral melanin pigmentations. The question may be asked if smokers' melanosis, like other melanotic lesions, may potentially develop into a malignant melanoma. We think not, for the following reasons:

1. Smokers' melanosis is one of the most commonly occurring oral lesions. Malignant melanoma is one of the rarest.
2. Smokers' melanosis, and pigmentations with an identical clinical and histologic appearance, are very seldom seen in the distal part of the maxillary alveolar ridge and palatal mucosa (I, II). These are the regions where most oral malignant melanomas are found (Chaudhry et al. 1958, Anneroth et al. 1973).

3. Malignant melanoma is twice as common in males as in females (Chaudhry et al. 1958). Smokers' melanosis is significantly more frequent in females as compared to males among individuals smoking up to 15 cig/day. Melanocytes in the female mucosa seem to be activated more easily by tobacco (I, II).

The possibility that smokers' melanosis may develop into a malignancy therefore seems remote. However, tobacco may still have an influence on the development of a melanoma. The present studies (IV, V) show tobacco to stimulate melanocytes. This points to the possibility that tobacco may also stimulate melanoma cells, and could perhaps influence the growth of pigmented tumor cells. The hypothesis is strengthened by the experimental finding that skin painting of rodents with coal tar does activate melanocytes, resulting in a clinical pigmentation, and promoting a downgrowth of melanocytes into the connective tissue. It also causes a pigmented tumor histologically similar to a malignant melanoma (Lipschütz 1924, Pawlowski & Lea 1983). Tobacco smoking also depresses the immune system and the defence against mutated cells (Koh et al. 1984, Phillips et al. 1985).

In the present studies (IV, VI), no morphologic alterations pointing to a harmful or degenerative influence on the epithelial melanin unit could be seen with the aid of light and electron microscopy. The melanocytes had normal dendrites and nuclei, and did not show multiple nuclei or abnormal numbers of dendrites, as in solar degenerated skin (Mitchell 1963). The normal morphology in smokers' melanosis also contrasted to the bizarre dendrites, the deeply indented nuclei, multiple cilia, and abnormal melanosomes seen in pigmented skin tumors (Maie & Schneider 1986). The development of melanosomes and their delivery to keratinocytes appeared to be normal. Melanocyte activation by nicotine and a following inactivation and reactivation showed the cells to react in a normal way (III, V). The keratinocytes handled melanosomes in a normal way, as evidenced by the degradation in suprabasal cell layers (Olson et al. 1979). The finding of enlarged MC in the oral mucosa is new, but is similar to observations in skin pigmentation after

UV-stimulation. The appearance of LMC is reported in normal human skin melanocytes (Drzewiecki 1979), and we have found no evidence that LMC in oral keratinocytes are pathologic products.

In the studied population oral melanin pigmentation is unusual in non-smokers. According to our hypothesis melanin is produced during the influence of toxic substances in order to serve as a detoxification system. Mostly the produced melanin is only visible in the microscope, but when it appears clinically visible as in smokers' melanosis it may be a sign of biologic alarm. As a similar cell reaction is also observed in other mammals (Iwata et al. 1981), this hypothesis may be relevant in a wider sense. The appearance of a new or increased melanin pigmentation may be an important indicator showing the living organism's reaction when exposed to environmental pollution.

SUMMARY AND CONCLUSION

The prevalence of oral melanin pigmentation, and the ultrastructure of clinically pigmented and non-pigmented gingiva was studied in smokers and non-smokers in northern and central Sweden. The morphologic studies were performed by light- and electron microscopy on unstimulated and stimulated melanocytes, melanin granules and melanin-containing keratinocytes. The diameter of melanosome complexes in keratinocytes was measured. The movement of melanosomes in the cytoplasm of amphibian skin melanocytes was studied by light microscopy and spectrophotometry during the influence of nicotine. The findings were:

1. Between 9.9 % and 14.9 % of the studies populations had oral melanin pigmentation. Among non-tobacco users 3.0 % were pigmented. In smokers between 21.9 % and 30.9 % were pigmented.
2. The pigment was histologically identified as melanin granules situated in the basal part of the epithelium.
3. More female smokers were pigmented than male smokers.
4. The pigmentation was more frequent among smokers who had been smoking for a long time than among those with shorter duration of smoking.
5. The pigmentation was less frequent among individuals who had stopped smoking. After 3 years of non-smoking, about 3 % of previously pigmented smokers were still pigmented.

6. Among pigmented individuals grouped according to cigarette consumption, a positive linear correlation was found between the number of pigmented individuals, and the number of cigarettes consumed ($r = 0.98$).
7. The morphology of melanocytes, melanin granules and melanin containing keratinocytes in clinically pigmented and non-pigmented areas of smokers was identical to that in pigmented non-smokers.
8. Melanosome complexes found in keratinocytes of pigmented individuals were larger than those earlier reported from human oral mucosa. Complexes larger than $1.0\mu\text{m}$ were observed.
9. In clinically pigmented and non-pigmented epithelium of smokers 83 % and 45 % respectively of basal keratinocytes contained melanin granules as compared to 4 % in non-pigmented non-smokers.
10. Dermal melanocytes in the toad *Bufo bufo* and the frog *Rana arvalis* were activated by nicotine as indicated by melanosomes moving out into dendrites giving a darkening of the skin. The morphologic changes were similar to those achieved by stimulation with MSH, theophylline or the β -receptor stimulator isoproterenol.
11. The melanocyte stimulation by nicotine was not blocked by propranolol. Nicotine is therefore not activating amphibian melanocytes via β -adrenergic receptors.

It is concluded that tobacco smoking rather than a genetical factor is the main cause for the oral melanin pigmentation observed in the studied population. The morphology of smokers' melanosis and the pigmentation observed in non-smokers is similar to that reported from skin pigmented by UV-irradiation. It is therefore concluded that smokers' melanosis is a non-specific reaction of the epithelial melanin unit caused by components in tobacco. Large melanosome complexes found in a high frequency are thought to be a normal expression of a high melanin production. Nicotine is shown to activate amphibian me-

lanocytes and is a possible co-factor to other causative agents in smokers' melanosis. It is suggested that the increased amount of melanin found in the oral epithelium of smokers acts as a trap for polycyclic compounds and free radicals in tobacco smoke entering the tissue, and for the free radicals produced in the tissue during the use of tobacco. The melanin-bound toxic products are expelled with the advancing keratinocyte in the corneal layer.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to:

Professor *Åke Larsson* my tutor and friend for your early support of my ideas, and for a still flourishing inspiration. Your positive criticism has made our mutual appointments inspiring events. Our wives Anita and Signe have contributed considerably to the efficiency and joy of these meetings.

Professor *Axel Bergenholtz* for introducing me to the field of research and scientific writing.

Dr *Lennart Hånström* for early support and fruitful discussions.

Dr *Tony Axéll* for stimulating talks during the preparation of our paper.

Dr *Gunnar Ronquist* for introducing me to the field of cyclic nucleotides, and for putting laboratory facilities at my disposal.

Dr *Torgny Alström* for giving me necessary laboratory equipment during amphibian studies.

Dr *Isser Brody* for putting the electron microscope at my disposal.

Dental hygienist *Kristina Berglund* for valuable help during the collection of amphibians.

Laboratory assistants *Karin Mårtensson*, *Eva Kreutz* and *Birgitta Sluga*, Department of Oral Pathology, and *Rose-Marie Jareholt*, *Maryanne Hedström* and *Ann-Britt Henriksson*, Department of Clinical Chemistry, for excellent technical assistance and a positive attitude to my work.

Mrs *Lillemor Hedin* for your skilful and prompt linguistic corrections.

Librarian *Anna Eckerdahl* for a very high quality service.

Hospital photographers *Öilme Eriksson* and *Alvar Johansson* for your positive attitude and extraordinary skill during the photographic production.

Head of Dentistry in Södermanland *John Erik Westborg* for your positive attitude towards the field of Science and for inspiring the development of Odontology in our county.

The investigations summarized in this thesis were financially supported by the *Swedish Medical Research Council* (project No. B86-24X-06872-03A), the *County Council of Södermanland* and the *Swedish Tobacco Company*.



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